Volume 211, number 1, 73–77 FEB 04354 January 1987

Inositol phospholipids are probably not the source of arachidonic acid for eicosanoid synthesis in astrocytes

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Received 14 November 1986

In astrocyte-enriched cultures of the rat cerebral cortex the Ca^{2+} ionophore A23187 provoked the breakdown of inositol phospholipids, the liberation of arachidonic acid and the release of prostaglandins E_2, $F_{2\alpha}$, I_2 and thromboxane A_2. However, agonists for receptors also coupled to inositol phospholipid metabolism in these cells failed to produce an increase in the release of both arachidonic acid and eicosanoids. Results suggest that the A23187-stimulated release of arachidonic acid and eicosanoids is caused by a phospholipase A_2-mediated attack on lipids other than the inositol phospholipids. Moreover, receptors linked to inositol lipid turnover are not involved in the control of eicosanoid release from astrocytes.

Astrocyte; Inositol phospholipid; Ionophore A23187; Arachidonic acid; Icosanoid; Receptor

1. INTRODUCTION

Eicosanoids have profound effects in the CNS where they appear to be involved in the modulation of behaviour, blood flow and neurotransmission [1]. It has recently been shown that at least one CNS cell type, the astroglia, possesses the capacity to synthesise and release these agents [2–5]. The close proximity of astrocytes to both neurons and the microvasculature in vivo suggests that eicosanoids released from these cells may play an important role in regulating a variety of neural functions. Consequently it is important that we understand the mechanisms involved in astroglial eicosanoid formation and the nature of the signals which control their release.

Recently, much attention has focused on the metabolism of the inositol phospholipids and the role played by their breakdown products in intracellular signal transduction [6–8]. However, these lipids also contain arachidonic acid (AA), the precursor of eicosanoid synthesis, and it has been proposed that their metabolism in response to a suitable stimulus may liberate AA for eicosanoid production [6,8]. We and others have demonstrated that eicosanoid release from astrocytes in primary culture can be stimulated by the Ca^{2+} ionophore A23187 [4,5]. Moreover, by measuring the accumulation of intracellular inositol phosphates we have also shown that A23187 induces a Ca^{2+}-dependent breakdown of inositol phospholipids in these cells [9]. Our aim, therefore, was to examine more closely the ionophore-stimulated metabolism of the inositol phospholipids and to determine whether this process is linked in some way to eicosanoid formation. In addition, we wished to establish whether other agents, such as agonists for receptors which are also coupled to inositol lipid turnover [9,10], are similarly involved in regulating astrocyte eicosanoid production.

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Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

2. MATERIALS AND METHODS

Astrocyte-enriched cell cultures of the newborn rat cerebral cortex were prepared and maintained as described [10,11]. Cells were seeded onto either 60 mm diameter dishes or 24-well plates and grown to confluence (14–18 days in vitro). Immunocytochemical characterisation of these cultures revealed that 90–95% of the cells were labelled with GFAP showing them to be astrocytes.

Cultures in 60 mm dishes were prelabelled for 24 h with 1 μCi/ml *myo*-[^{3}H]inositol (15 Ci/mmol, Amersham, final concentration 10 μM) to achieve maximum labelling of lipids [9]. The stimulus-induced formation of total [^{3}H]inositol phosphates over a 60 min incubation period in the presence of 5 mM Li^+ was assessed as described [9]. In some experiments an analysis of the individual [^{3}H]inositol phosphates formed during short exposure periods (5 s–10 min) was undertaken. These incubations were carried out in the absence of Li^+ and the material from four cultures pooled for each determination. The column separation of [^{3}H]inositol trisphosphate (IP_3), bisphosphate (IP_2) and monophosphate (IP_1) was performed by the method of Brown et al. [12]. The A23187-evoked change in the incorporation of [^{3}H]inositol into individual inositol lipids was determined by separation of the labelled lipids by thin-layer chromatography [13]. The material from two cultures was pooled and applied to chromatography plates together with lipid standards obtained from Sigma. The R_f values for each lipid were as follows: phosphatidylinositol (PI, 0.45), phosphatidylinositol 4-phosphate (PIP, 0.25) and phosphatidylinositol 4,5-bisphosphate (PIP_2, 0.19).

Cultures in 24-well plates were used to determine either the liberation of ^{14}C from cells prelabelled with [^{14}C]AA or the release of eicosanoids. For the former, cultures were labelled for 18 h with 0.1 μCi/ml [^{14}C]AA (59.6 mCi/mmol, Amersham) which resulted in approx. 20% of the radiolabel being incorporated. Cultures were washed twice with Earles balanced salt solution (EBSS) prior to incubation for 30 min with either A23187 or other agents. The ^{14}C released into the incubation medium was determined by liquid scintillation counting. In the latter experiments, cultures were washed with EBSS prior to a 30 min exposure to a stimulus. The incubation medium was collected, extracted with double distilled ethyl acetate and the presence of eicosanoids detected by radioimmunoassay. Four eicosanoids were assayed for using antisera (Capell Laboratories) of high serological specificity against prostaglandins E_2 (PGE_2), $F_{2\alpha}$ ($PGF_{2\alpha}$), 6-oxo-prostaglandins $F_{1\alpha}$ (6-oxo-$PGF_{1\alpha}$) and thromboxane B_2 (TXB_2), the latter being the stable metabolites of prostaglandin I_2 (PGI_2) and thromboxane A_2 (TXA_2), respectively. Labelled and unlabelled eicosanoids were purchased from NEN and Sigma, respectively, and assays were carried out according to protocols supplied by Capell Laboratories as described [14].

3. RESULTS AND DISCUSSION

A23187 stimulated the liberation of ^{14}C from cells prelabelled with [^{14}C]AA and the accumulation of [^{3}H]inositol phosphates in cells prelabelled with [^{3}H]inositol in a dose-dependent manner (fig.1). Maximal effects for these responses were obtained with 10 μM A23187 and represented 4–5-fold increases over basal. Four eicosanoids were assayed for and detected in the conditioned medium. The dose-response curve for A23187-stimulated release of TXA_2 is shown in fig.1, similar curves were obtained for PGE_2, $PGF_{2\alpha}$ and PGI_2. Again maximal TXA_2 release was obtained

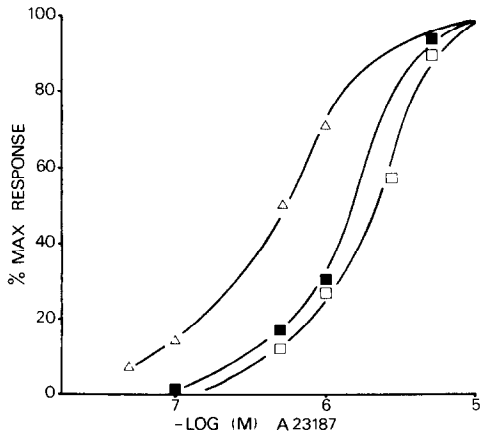

Fig.1. Concentration-dependence of A23187-stimulated release of TXA_2 (△), ^{14}C liberation (■) and [^{3}H]inositol phosphate accumulation (□) in astrocyte-enriched cultures. Values are means (SE < 15%) from 4–6 determinations.

Table 1

Time course of A23187-evoked changes in the recovery of ^3H in inositol, inositol phospholipids and inositol phosphates

Incubation time	^3H recovered (dpm × 10^{-2})						
	Inositol	PI	PIP	PIP_2	IP_1	IP_2	IP_3
0	85395.2 ± 1526.4	2128.7 ± 68.2	15.6 ± 2.7	14.7 ± 4.0	4.6 ± 0.6	11.0 ± 1.7	3.4 ± 0.8
5 s	61112.8 ± 6359.8[a]	1732.1 ± 160.4[a]	30.5 ± 4.2[a]	12.7 ± 1.4	6.0 ± 0.6[a]	17.5 ± 2.4[a]	4.8 ± 1.1
30 s	69707.2 ± 3919.2[a]	1834.2 ± 187.2	25.9 ± 2.7[a]	19.4 ± 3.7	6.8 ± 0.8[a]	21.8 ± 3.3[a]	5.3 ± 0.9
60 s	55041.6 ± 6105.0[a]	1703.3 ± 107.8[a]	26.0 ± 2.7[a]	13.2 ± 4.7	7.3 ± 0.6[a]	18.3 ± 1.8[a]	4.3 ± 0.3
10 min	60686.4 ± 6095.4[a]	1765.5 ± 108.1[a]	26.9 ± 3.3[a]	10.6 ± 3.3	14.9 ± 0.9[a]	20.9 ± 1.8[a]	3.8 ± 0.4

[a] Significantly different from results at zero time ($P < 0.05$) by Mann-Whitney U-test

Astrocyte-enriched cultures were prelabelled with [^3H]inositol then exposed to A23187 (5 μM) for the periods indicated. Values are means ± SE from 4 determinations

with 10 μM ionophore and corresponded to a 15-fold increase over basal levels. A23187 appeared to be more effective (EC_{50} 0.5 μM) in promoting eicosanoid release than in liberating either ^{14}C (EC_{50} 1.5 μM) or the accumulation of [^3H]inositol phosphates (EC_{50} 2 μM). PGE_2 was found to be the most abundant eicosanoid released followed by $PGF_{2\alpha}$ and TXA_2 which were released in almost equal amounts. Relatively little PGI_2 was found in the conditioned medium due to the fact that it is synthesised largely in meningeal fibroblasts only a small population (5–10%) of which contaminate our astrocyte cultures [5]. The other eicosanoids were derived entirely from the astrocytes in these cultures (Jeremy et al., unpublished).

The Ca^{2+} ionophore A23187 clearly promotes the breakdown of inositol phospholipids in astrocytes. Previous studies have shown this response to be entirely dependent on the presence of extracellular Ca^{2+} [9], suggesting the involvement of a Ca^{2+} requiring phospholipase. To determine which inositol lipids were metabolised in response to A23187 treatment we examined the change in labelling of the lipids and the accumulation of their labelled breakdown products (IP_3, IP_2 and IP_1). Table 1 shows that in control cultures prelabelled with [^3H]inositol for 24 h, the bulk (98%) of the label was incorporated into PI, the remainder being equally distributed in the phosphoinositides PIP and PIP_2. Incubation with A23187 produced no marked or consistent change in either the labelling of PIP_2 or the accumulation of IP_3 in these cells (table 1). However, there did appear to be an increase in the turnover of PIP. This was characterised by a rapid and sustained increase in both the accumulation of IP_2 and the recovery of ^3H in PIP. There was also a small (20–30%) but significant loss of label from PI and the pool of free inositol (table 1) which may have been the result of the need to replenish the pool of PIP. However, a direct A23187-induced breakdown of PI cannot be excluded because even after a 5 s incubation period a significant increase in IP_1 accumulation was evident.

The finding that A23187 does not affect the metabolism of PIP_2 but primarily attacks PIP is in agreement with previous studies performed on other tissues [15,16]. These data confirm that the phospholipase C-mediated breakdown of PIP_2 is not dependent on an increase in intracellular Ca^{2+}. Moreover, they also suggest that the ^{14}C liberated from [^{14}C]AA-labelled astrocytes was not the result of diacylglycerol formation from this lipid. Nonetheless, these results do not rule out the possibility that AA may be liberated from either PIP and/or PI via the activation of a Ca^{2+}-dependent phospholipase C [17].

In a number of tissues activation of muscarinic and α_1-adrenergic receptors leads to changes in the synthesis and release of eicosanoids [1,18–20]. Astrocytes also possess these receptors and they are coupled to membrane inositol phospholipids [9,10]. However, the results presented in table 2 clearly show that although agonists for these receptors produced large increases in the accumulation of [^3H]inositol phosphates they were without effect

Table 2

Effect of A23187 and receptor agonists on [^{3}H]inositol phosphate accumulation, [^{14}C]AA liberation and TXA_2 release

Additions	[^{3}H]Inositol phosphates (dpm)	[^{14}C]AA (dpm)	TXA_2 (ng/ml)
None	1656 ± 133	5226 ± 471	2.5 ± 0.1
A23187	5825 ± 152	28986 ± 5211	28.0 ± 0.6
Carbachol	6877 ± 236	4455 ± 102	3.4 ± 0.6
Noradrenaline	11864 ± 1611	4305 ± 45	2.2 ± 0.2

Astrocyte-enriched cultures were prelabelled with either [^{3}H]inositol or [^{14}C]AA then incubated with 10 μM A23187, 1 mM carbachol or 1 mM noradrenaline for 60 min (inositol phosphates) or 30 min (AA and TXA_2 release). Values are means ± SE from 4–6 determinations

Table 3

Effect of mepacrine on A23187-evoked [^{3}H]inositol phosphate accumulation and TXA_2 release

Additions	[^{3}H]Inositol phosphates (dpm)	TXA_2 (ng/ml)
None	1406 ± 71	6.2 ± 0.2
A23187	7102 ± 460	40.0 ± 1.4
A23187 + mepacrine	6427 ± 60	14.6 ± 0.4

Astrocyte-enriched cultures were exposed to 10 μM A23187 for 60 min in the presence or absence of 10 μM mepacrine. Values are means ± SE from 4 determinations

on either the liberation of ^{14}C from [^{14}C]AA-labelled cultures or the release of TXA_2. In addition, whether they were used in combination or with a submaximal dose of A23187, no agonist-induced change in the latter two responses was observed (not shown). Work carried out on both a glioma cell line and on primary astrocytes shows that agonists such as those used here do liberate AA from lipids although the response is very transient and occurred inside the cell only [21,22]. These authors also reported the generation briefly of an unidentified lipoxygenase metabolite, but the levels of PGI_2, $PGF_{2\alpha}$, PGE_2 and PGD_2 remained unchanged. Our previous studies have shown that receptor activation induces the breakdown of PIP_2, however, a direct Ca^{2+}-dependent breakdown of PIP and/or PI was also suggested [9]. It would seem therefore that any AA liberated from these lipids in the form of diacylglycerol was recycled via phosphatidate and not used for eicosanoid synthesis. In this regard our data are in agreement with other studies on synaptosomes [23] and GH_3 pituitary cells [24].

These findings raise the possibility that even in response to treatment with A23187, the inositol phospholipids are not the source of AA for eicosanoid synthesis in astrocytes. It is generally accepted that the metabolism of these lipids is mediated by phospholipase C although it has been suggested that PI may also undergo attack by phospholipase A_2 [25]. Table 3 shows that whereas A23187-stimulated [^{3}H]inositol phosphate accumulation was unchanged, the ionophore-evoked increase in TXA_2 release was markedly reduced (~70%) by mepacrine an apparently selective inhibitor of phospholipase A_2 [26]. We interpret these results as indicating that eicosanoid release from astrocytes in response to A23187 is the result of a phospholipase A_2-mediated attack not on inositol phospholipids but on other AA-containing lipids such as phosphatidylcholine and/or phosphatidylethanolamine as appears to be the case in many other cells [24,27].

In conclusion, the use of A23187 has revealed that astrocytes have the ability to mobilise AA and to synthesise and release eicosanoids. It would appear that although the ionophore provokes the metabolism of inositol phospholipids these do not

constitute the source of AA for eicosanoid synthesis. Moreover, agonists for receptors which also induce inositol lipid breakdown are without effect on either AA or eicosanoid release, any diacylglycerol liberated upon stimulation is probably recycled. Thus, the physiological control mechanisms which regulate astrocyte eicosanoid formation remain to be resolved.

ACKNOWLEDGEMENTS

We thank members of the Brain Research Group for useful discussion. This work is supported in part by an SERC project grant to S.M.

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